The Mechanism of Sex Determination in *Rumex acetosella*

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Summary. Cytogenetic studies were made with particular emphasis on the sex-determining mechanism in *Rumex acetosella* ($6x = 42$) and its hybrids (F_1, F_2, BC_1 and BC_2) with *R. hastatulus* (synthetic $4x = 16 = 4A + 4X = ♀$ and $4x = 18 = 4A + 2(XY_1Y_2) = ♂$). *Rumex acetosella* was almost strictly dioecious with 50:50 male and female. Breeding tests revealed that the males were heterogametic. The longest chromosomes (S), usually two, are the sex chromosomes of this hexaploid species. The S chromosomes are homomorphic in both male and female. The sex chromosome: autosome ratios, and the strong epistatic male effect of the S^M chromosome in the polyploid dioecious species and in the hybrids, are evidence of an X/Y *Melandrium* type sex-determining mechanism controlled by a single pair of homomorphic sex chromosomes. Thus, the sex chromosome formula of the males was $S^F S^M$ and that of females was $S^F S^F$. The present approach is a new method for resolving the sex-determining mechanism in a dioecious species.

Introduction

An X/A balance determines sex in the diploid dioecious species of the subgenus *Acetosa* (Ono, 1935; Yamamoto, 1938; Smith, 1963). Confirming Muller's (1925) generalisation that the X/A balance in sex determination precludes the occurrence of polyploidy, polyploid forms do not exist in the dioecious species of this sugbenus. *Rumex paucifolius*, of the same subgenus, karyotypically resembles *Acetosella* species (Smith, 1968), but does exist in polyploid forms and is presumed to have an X/Y sex-determining mechanism (Löve and Sarkar, 1956). On the other hand, all four species of the subgenus *Acetosella* are dioecious and exist in a polyploid series, one at each ploidy level from diploid to octoploid. Löve (1944) suggested that an X/Y balance determines sex in the subgenus *Acetosella*. The females are homogametic with 2, 4, 6 and 8 X chromosomes, while the males are heterogametic with a single Y and 1, 3, 5 and 7 X chromosomes, in the $2x$, $4x$, $6x$ and $8x$ species, respectively. No critical evidence has been put forward to support the proposed X/Y balance.

We (Singh, 1964) have studied in detail the cytogenetic nature, with particular emphasis on sex determination, of *Rumex acetosella* subgenus *Acetosella*. It was found that *R. acetosella* is a segmental allohexaploid with the somatic chromosome number $6x = 42$ (Singh, 1968). One to four, usually only two, homomorphic sex chromosomes were found in both males and females, even though the species is hexaploid. Six autosomes of one type were recorded. The "extra" sex chromosomes were differentiated into autosomes. The present paper suggests the sex-determining mechanism of the dioecious hexaploid *R. acetosella* as substantiated by experimental evidence.

Materials and Methods

Eight different cultures of *Rumex acetosella* obtained from eight widely separated localities in North America, South America and Germany, and the different hybrids (F_1, F_2, BC_1 and BC_2) of North Carolina races of *R. acetosella* and *R. hastatulus*, were studied. *Rumex hastatulus* is an annual dioecious weedy coloniser of the subgenus *Acetosa* and had females, $2x = XX + 6$, and males, $XY_1Y_2 + 6$, and synthetic $4x = 16$, 17 and 18 chromosomes. As noted earlier, an X/A balance determines sex in *R. hastatulus*. The somatic chromosomes of each plant were studied in acetic orcein squashes of root tips pretreated with 8-hydroxyquinoline. At flowering, the flowers of each inflorescence were assessed as pistillate or staminate by macroscopic examination.

Observations and Discussion

The detailed karyotype of *R. acetosella* has been reported elsewhere (Singh, 1964). The longest chromosomes which are morphologically similar in the males and females are the sex (S) chromosomes of this species (evidence presented in a later section). Table 1 presents the distribution of S chromosomes and male and female frequencies in *R. acetosella*. Two S chromosomes were found in more than 64 per cent of the plants analyzed. The distribution pattern of one, three and four S chromosomes in the population is not random, as revealed by Table 2. It indicates that, barring the aneuploid variations, the different populations, from Argentina, Arkansas, Germany and elsewhere, have varying levels of differentiation of the sex chromosomes with a tendency towards their disomic balance. Table 1 also shows that *R. acetosella* is almost strictly dioecious with a 50:50 proportion of males and females.

Table 1 reveals that both males and females have identical combinations of S chromosomes. The numerical variation in S chromosomes, within and between localities, is similar in both sexes. As regards morphological differences between the chro-

Table 1. *Distribution pattern of S chromosomes and male and female frequencies in R. acetosella*

Source	No. of S chromosomes and the sex								% of males	% of females	Total M & F	Total plants examined cytologically
	1		2		3		4					
	M	F	M	F	M	F	M	F				
North Carolina	0	1	8	9	0	0	0	0	44.44	55.56	18	27
Tennessee	0	0	8	6	0	0	0	0	57.14	42.86	14	21
Arkansas	0	0	4	4	3	2	0	0	53.15	46.85	13	34
Colorado	0	0	5	4	1	0	0	0	60.00	40.00	10	26
Montana	0	0	1	1	0	0	0	0	50.00	50.00	2	20
Argentina	2	4	2	1	0	0	0	0	44.44	55.56	9	23
Chile	0	0	1	0	0	2	0	0	33.33	66.67	3	19
Germany	0	0	0	0	0	0	4	0	100	00.00	4	29
Total	2	4	29	25	4	4	4	0	52.78	48.22	73	204

mosomes of any class, male or female, nothing could be detected. Thus, males and females are cytologically similar.

It is interesting to note that numerical variation in S chromosomes was not a monopoly of any particular population. The converse is not true; only two S chromosomes were found in all the plants studied from Tennessee. No plant was found to be nullisomic for S, suggesting that at least one S chromosome was essential for the existence of a plant either as a male or a female. On the other hand, trisomic-S and tetrasomic-S constitution was compatible with normal growth and development.

The sex-determining balance has been studied primarily by correlating the balances among autosomes, X chromosomes and Y chromosomes with the sex expressed in normal diploids, synthetic aneuploids and polyploids. This approach has not been applied hitherto in recognising the sex chromosomes and in resolving the sex-determining mechanism in the polyploid dioecious Rumices. Westergaard (1958) pointed out the following three consecutive steps which must be taken in the analysis of the genetics of sex determination in a given species: first, establish which sex is heterogametic; second, localize the sex-determining gene complexes in the sex chromosomes and their interactions with the autosomal genes; third, analyze the mode of action of the individual genes concerned. The first two steps were analyzed in the present study by a method, not reported previously, which is an addition to the seven methods outlined by Correns, Westergaard and Janick (Burnham, 1962).

The approach was as follows. Hybrids, F_1, F_2, $B C_1$ and $B C_2$, were produced, involving *R. hastatulus* (4 x = 16, 18 with the sex chromosome formula 4 A : 4 X = ♀ and 4 A : 2(X Y_1 Y_2) = ♂) with an X/A balance, on one hand, and *R. acetosella*, whose cytogenetic basis of sex determination is unknown, on the other hand. The male of *R. hastatulus*, as in other dioecious Rumices, is heterogametic. The karyotype of each hybrid was studied critically and correlated with the sex expressed. Table 3 presents the chro-

Table 2. *Sex chromosome frequencies in R. acetosella*

No. of sex chromosome	Frequency	Percentage	Percentage of the chromosome combination derived from a single locality
1	18	8.82	7.14 (Argentina)
2	131	64.22	—
3	27	13.24	6.86 (Arkansas)
4	28	13.72	12.75 (Germany)
Total	204	100	

mosomal and genic balance and the sex expressed in the hybrids.

F_1: 46 plants were pistillate and 30 were staminate with 2 n^* = 29 (8 h + 21 a c) and the sex chromosome formula 2 X h + 1 S. No differences could be detected in the morphology of the somatic chromosomes of males and females.

The F_1 sex ratio, 46 females: 30 males, is significantly different from the expected 38:38 ($\chi^2 \geq 2.71$ $\leq$ .01). The sample of 76 plants is not large enough to conclude whether the higher proportion of females is real. In the strictly unisexual and chromosomally balanced plants with 2 n = 29 = 8 h (2 A + 2 X) + 21 a c (20 a + S), both males and females had the female genic balance of *R. hastatulus* plus one S chromosome of *R. acetosella*. No cytological difference could be detected in the genome ac chromosomes of male and female F_1 hybrids. This suggests that the males of *R. acetosella* are heterogametic and produce morphologically similar male and female gametes in equal proportions (the sex ratio in *R. acetosella* was 50:50), and consequently gave males and females in the F_1 hybrids in nearly the same ratio. Thus, the male factors of genome ac are epistatic to the female factors of *R. acetosella* as well as to the female balance of the genome h. The female factors of genome ac are additive to the female genic balance of genome h.

* The somatic chromosome number of the hybrids has been denoted by 2 n even though they contained multiples of the basic chromosome number or varying aneuploid chromosome combinations of the two species.

Table 3. *Chromosomal and genic balance and the sex expressed in the hybrids of R. hastatulus and R. acetosella*

Generation	Parents and their genic balances		Progenies and their genic balances		Female: Male expected 1:1 observed
	Female	Male	Female	Male	
F_1	$(4\,A + 4\,X)\,h$	$(40\,a + S^F\,S^M)\,a\,c$	$2\,A + 2\,X,\ + 20\,a + S^F$	$2\,A + 2\,X,\ + 20\,a + S^M$	46:30
F_2	$(2\,A + 2\,X,\ + 20\,a + S^F)\,F_1$	$(2\,A + 2\,X + 20\,a + S^M)\,F_1$	$2\,A + 2\,X,\ + 20\,a$	$2\,A + 2\,X,\ + 20\,a + S^M$	3:10*
			$2\,A + 2\,X,\ + 20\,a + S^F$	$2\,A + 2\,X,\ + 20\,a + S^F\,S^M$	
$B\,C_1$	$(2\,A + 2\,X,\ + 20\,a + S^F)\,F_1$	$(4\,A + 3\,X + Y_1\,Y_2)\,h$	$3\,A + 3\,X,\ + 10\,a$	$3\,A + 2\,X + Y_1\,Y_2,\ + 10\,a$	19:24*
			$3\,A + 3\,X,\ + 10\,a + S^F$	$3\,A + 2\,X + Y_1\,Y_2,\ + 10\,a + S^F$	
	$(4\,A + 4\,X)\,h$	$(2\,A + 2\,X + 20\,a + S^M)\,F_1$	$3\,A + 3\,X,\ + 10\,a$	$3\,A + 3\,X,\ + 10\,a + S^M$	
$B\,C_2$	$(3\,A + 3\,X,\ + 10\,a)\,B\,C_1$	$(4\,A + 3\,X + Y_1\,Y_2)\,h$	$3 - 4\,A + 3 - 4\,X,\ + 4 - 6\,a$	$3 - 4\,A + 2 - 3\,X + Y_1\,Y_2,\ + 4 - 6\,a$	46:46*
	$(4\,A + 4\,X)\,h$	$(3\,A + 3\,X,\ + 10\,a + S^M)\,B\,C_1$	$3 - 4\,A + 3 - 4\,X,\ + 4 - 6\,a$	$3 - 4\,A + 3 - 4\,X,\ + 4 - 6\,a + S^M$	
	$(4\,A + 4\,X)\,h$	$(3\,A + 2\,X + Y_1\,Y_2,\ + 10\,a)\,B\,C_1$	$3 - 4\,A + 3\,X,\ + 4 - 6\,a$	$3 - 4\,A + 2\,X + Y_1\,Y_2,\ + 4 - 6\,a$	

* Andromonoecious intersexes have been included in the male class.

A = Complete set of autosomes a = an autosome
S^M = Male determining S chromosome h = R. *hastatulus* chromosomes
S^F = Female determining S chromosome $a\,c$ = R. *acetosella* chromosomes

F_2: Twelve plants were strictly unisexual (three pistillate plus nine staminate) and one was an andromonoecious intersex. The diploid chromosome numbers varied from 26 to 30. Plants with the sex chromosome formula $1\,X + 2\,S$ and $2\,X + 2\,S$ were always males, while those with the formula $1\,X + 1\,S$ and $2\,X + 1\,S$ were either males or females, in 1:1 ratio. All F_2 hybrids had at least one, and a maximum of two, S chromosomes.

$B\,C_1$: The data presented in the $B\,C_1$ section give insight to the problem of the sex-determining mechanism of hybrids. The 14 progenies of the cross $F_1 = 2\,n$ 29 $(2\,A + 2\,X + 1\,S)$ ♀ × R. *hastatulus* $4\,x = 17$ $(4\,A + 3\,X + Y_1\,Y_2)$ ♂ in general had similar autosomes but differed in their sex and sex chromosome formula. Six plants were strictly staminate with $2\,n = 21$ to 23 and the sex chromosome formula $2\,X + Y_1\,Y_2 + 1\,S$ and $2\,X + Y_1\,Y_2 + 0\,S$. Seven plants were strictly pistillate with $2\,n = 18$ to 23 and the sex chromosome formula $3\,X + 1\,S$ and $3\,X + 0\,S$. The S chromosome did not have a decisive role in these progenies as its presence or absence did not make any difference to the sex expression. The F_1 females produced similar gametes, genetically, and, thus, are homogametic in the strict sense $(1\,A + 1\,X + 1\,S$ are genetically similar to $1\,A + 1\,X + 0\,S)$. The $B\,C_1$ plants are males and females in an approximately 50:50 ratio, because the male parent, R. *hastatulus*, is heterogametic. The $Y_1\,Y_2$ chromosomes of genome h turn the balance $2\,X{:}3\,A + 1\,S$ and $2\,X{:}3\,A + 0\,S$, towards male expression. The female factors of ac genome are hypostatic to $Y_1\,Y_2$ of h genome.

The reciprocal cross R. *hastatulus* $4\,x = 16$ $(4\,A + 4\,X)$ ♀ × F_1 ♂ behaved differently. Twelve plants were strictly pistillate with $2\,n = 20$ to 26 and the sex chromosome formula $3\,X + 0\,S$ and $4\,X + 0\,S$. On the other hand, 16 were strictly staminate with $2\,n = 20$ to 28 and the sex chromosome formula $2\,X + 1\,S$ and $3\,X + 1\,S$. In this particular cross, the S chromosome made the individual male wherever it was present. Two andromonoecious intersexes appeared in $B\,C_1 \cdot B\,C_1$, R. *hastatulus* $4\,x = 16$ $(4\,A + 4\,X)$ ♀ × F_1 $2\,n = 29$ $(2\,A + 2\,X + 1\,S)$ ♂, produced males and females in approximately equal proportions. The female parent was homogametic and the male parent was heterogametic. The two types of gametes, $(1\,A + 1\,X + 1\,S)$ and $(1\,A + 1\,X + 0\,S)$, produced by the males, are male and female determining, respectively, because the presence of the single S results in male sex expression. The S chromosome from the male F_1 hybrid carries the male determiners and the converse is also true: these hybrids are male because they contain the male determining S chromosome. The male factors (S^M) are epistatic to the $1\,X{:}1\,A$ female balance of R. *hastatulus*. If S^M is not present in the hybrids, the genic balance of genome h determines the sex expression, irrespective of anything else contributed by the genome ac. Thus, the S chromosome is the sex chromosome of R. *acetosella*.

$B\,C_2$: Forty-three flowering plants were obtained from crosses between $B\,C_1$ ♀ and R. *hastatulus* $4\,x = 17$ or 18 ♂. Fifteen were strictly staminate with $2\,n = 19$ to 21 and the sex chromosome formula $2\,X + Y_1\,Y_2 + 0\,S$ and $3\,X + Y_1\,Y_2 + 0\,S$. Twen-

ty-two were strictly pistillate with $2n = 17$ to 22 and the sex chromosome formula $3X + 0S$ and $4X + 0S$. It is evident that if $Y_1 Y_2$ were present the individual was male, if absent the individual was female, irrespective of any other chromosome.

Three exceptionally interesting intersexes appeared in this generation. It is important to note that the appearance of the intersexes increased with advancing hybrid generations.

The reciprocal, *R. hastatulus* $(4x = 16) ♀ \times B C_1 ♂$, produced a single plant which was pistillate with $2n = 19 (3A + 3X, + 7a + 0S)$ chromosomes.

One plant which was obtained from the cross *R. hastatulus* $2x = 8 (2A + 2X) ♀ \times B C_1 ♂$ was strictly staminate with $2n = 15 (8h + 7ac)$, and the chromosome balance $2X:2A + 1S^M$ chromosome caused maleness, even though a balanced diploid set of female *R. hastatulus* was present.

The facts revealed by the $B C_2$ progenies, with regard to their sex-determining mechanisms, are similar to those described under $B C_1$. It was apparent from the $B C_1$ results that the S chromosome is the sex chromosome of *R. acetosella*. This statement is further strengthened by the fact that in the almost balanced triploid to tetraploid genetic back-ground of genome h, $1a$ to $12a$ genome ac chromosomes from all the groups (D to L) (Singh, 1964), except the S chromosomes, did not alter the genic balance for sex determination of *R. hastatulus* and sex expression was determined by genome h balance. When the S^M chromosome accompanied the genome ac chromosomes, the plants were male irrespective of genome h balance. This eliminates the possibility of any other chromosomes (D to L) of *R. acetosella* being involved in sex determination, and thus, these could be referred to as autosomes. What the exact nature of S^F chromosomes in the hybrids is (as regards sex determination) is not known except that it is compatible with genome h balances.

The study of the sex-determining mechanisms of backcrosses will explain the observations made in F_1 and F_2 progenies. The 1 female: 1 male ratio is quite compatible with the fact that the *R. acetosella* male is heterogametic. The hybrid plants which had both the S chromosomes of the male parent ($S^F S^M$) (probably resulting from nondisjunction) were always staminate because S^M is epistatic to S^F.

The distribution pattern of S chromosomes and its association with sex expression in the hybrids has established that the S chromosome is the sex chromosome of *R. acetosella*. The S chromosomes are homomorphic, but they carry the female and male determiners.

The sex factors for female and male can be symbolically represented as S^F and S^M, respectively. It is concluded from Table 1 that at least one S chromosome is necessary for survival. Both females and males can have a single S chromosome and be phenotypically identical to plants which have two S chro-

mosomes. The sex chromosome-autosome balances $S^F S^M$:40 a and S^M:40 a or 41 a produce morphologically identical males. Similarly, the ratios 2 S:40a and S^F:40 a or 41 a produce morphologically identical females. In the hybrid background, 1 S^M:6 a, 1 S^M:10 a, 1 S^M: 20 a and $S^F S^M$:20 a ratios of S chromosome and autosomes of the ac genome apparently had no effect on male sex expression, as all the males produced under these balances were strictly unisexual, irrespective of the strictly female balances (2 X:2 A, 3 X:3 A) of the *R. hastatulus* complement. In other words, $S^M = = S^M S^F = ♂$, and $S^F = S^F S^F = ♀$, as regards their sex expressing potentiality. It is concluded that the presence of an S^M chromosome produces a male and its absence allows the development of femaleness. Not only the absence of the S^M chromosome, but also the presence of the S^F chromosome, is probably necessary for female sex expression, as indirectly shown by the presence of at least one S^F chromosome in all the females observed. The autosomes apparently do not contain genes which impair the potentialities of the S^M chromosomes. The inferences based on hybrids are open to certain criticisms, however, for it is hard to decide exactly what role the cytoplasmic factors and interactions between autosomal and sex genes of both the species play in sex determination.

Rumex acetosella was not strictly dioecious. One plant out of the 92 analyzed had three flowers with varying degrees of pistillate development among 20 male flowers examined. This andromonoecious intersex had $6x = 42 (40a + 1S + 1B*)$ chromosomes. Ono (1930) collected several *R. acetosella* intersexes in Japan. He examined the meiotic division in one of them. Twenty bivalents plus one univalent were observed at first metaphase. He failed to determine whether all the intersexes collected had 41 somatic chromosomes. Ono did not give any cytogenetic explanation for the rare occurrence of the intersexes. Another andromonoecious intersex with $6x = 42$ chromosomes was found by Löve (1944). Löve assumed that the intersexes are the results of the accumulation of female-determining genes in the autosomes. Gynomonoecious intersexes have not been reported to occur in *R. acetosella*. The occurrence of the andromonoecious intersexes, however, suggests the bisexual potentiality of the diploid male genotype (Goldschmidt, 1955).

If the species *R. acetosella* was strictly dioecious, its sex determining mechanism could be explained by a monofactorial gene pair causing maleness in the heterozygous state (M/F) and femaleness in the "recessive" homozygous (FF) condition (M factor being epistatic to F factor). But this is not the case. The rare occurrence of andromonoecious intersexes of varying strength (some plants have male flowers predominantly, with rudimentary pistils in a few of

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* B chromosome is homologue of S chromosome but slightly smaller in size.

the male flowers, while some plants have functional hermaphroditic flowers) in the natural population cannot be explained on such a simple genetic model.

The sex chromosome: autosome balance ratios, $2 S^F : 6 A$ or $1 S^F : 6 A$, are associated with strict femaleness in *R. acetosella*. Probably, such a case of genic balance has not been reported previously in any dioecious organism. Interpreting Löve's (1944) observations on *R. angiocarpus*, it can be stated that a one-to-one relationship between the X chromosomes and the basic set of autosomes is associated with strict femaleness (similar to the cases found in *R. hastatulus*, in *R. acetosa* and its close relatives, in *Melandrium*, and in *Drosophila*. Considering the segmental allopolyploid origin of *R. acetosella* from the diploid *R. angiocarpus*, and the relative morphological constancy of autosomes in the former species, it may be postulated that *R. acetosella* autosomal genes are qualitatively similar to, but quantitatively different from those present in *R. angiocarpus*. If this statement is true, the unusual balance between the S^F chromosomes and autosomes of *R. acetosella* can be visualized to a certain extent, but the quantitative differences cannot be ignored. Critical differences between the modes of sex determination in *R. acetosella* and other well-studied dioecious species became evident when the chromosome conditions associated with maleness and intersexuality were compared. *Rumex acetosella* resembles *Melandrium* in that males and intersexes occurred only when the S^M (Y in *Melandrium*) chromosome was present, irrespective of any other chromosomes. (The presence of the S^M chromosome can only be inferred from breeding tests, since S^M and S^F chromosomes are morphologically identical; S^M chromosome is always inherited from the male parent.)

The S^F chromosome and autosome ratio variations (which do not have a striking effect on the sex expression) suggest that apparently the basic X/A balance has no effect on sex determination in *R. acetosella*. A similar and well documented case was studied in *Melandrium* (Warmke, 1946; Westergaard, 1940, 1958). The rare occurrence of andromonoecious intersexes is tentatively ascribed to the segregation of certain minor sex-modifying autosomal genes. A complete absence of gynomonoecious intersexes suggests that the S^M chromosome is necessary for androecium development and must also inhibit gynoecium development since the plants without S^M chromosomes are always females.

In the light of these facts, the cytogenetic mechanism of sex determination in *R. acetosella* may be stated as follows: A single pair of homomorphic sex chromosomes (the S^F chromosomes and the S^M chromosomes) are primarily responsible for sex expression. The S^M chromosome plays a decisive part in determining sex, probably by inhibiting the female potential of the S^F chromosome (possibly of certain autosomes, also) and promoting and completing male development. The female potentials of S^F chromosomes are only expressed if S^M is absent. The autosomes probably do not play a decisive role in sex determination. Whether the male and the female potentialities of the S^F and S^M chromosomes are controlled by a few major genes or a complex of minor genes is unknown. The present case of sex determination studied appears to be unique in having only two sex chromosomes in the hexaploid species. The sex determination in *R. acetosella* is based on an X/Y *Melandrium* system.

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